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CADASIL:
A PURE MODEL FOR STUDYING CEREBRAL SMALL VESSEL DISEASE

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CADASIL: A pure model for studying cerebral small vessel disease

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ABSTRACT

Cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy (CADASIL) is caused by a mutation on the *NOTCH3* gene. The pathological driver behind this disease is the loss of vascular smooth muscle cells (VSMCs) in small blood vessels and subsequent fibrotic thickening of the vessel, causing stenosis. Although a great deal of knowledge has been accumulated through CADASIL research, more information is needed to fully grasp the pathological mechanisms as well as understand disease progression. We have focused on the effects of *NOTCH3* mutation on VSMCs and identified several novel actors in disease progression.

In our initial study, we investigated factors playing a role in the VSMC proliferative rate. We found that VSMCs carrying a CADASIL mutation express higher levels of TGFβ. This elevated cytokine secretion by CADASIL VSMCs had an inhibitory proliferative effect on not only these cells, but also cells in their vicinity. This phenotype was rescued by adding neutralizing TGFβ antibody to the growth media, which increased CADASIL VSMC proliferation rate to that of control VSMCs, while having a minimal effect on control VSMC proliferation rate.

One of the initial events in CADASIL disease progression is NOTCH3 aggregation and accumulation. In our next study, we focused on degradation of aggregated NOTCH3 via autophagy processes. After confirming that NOTCH3 is aggregated and accumulated in our cell line, we further tested the autophagy process in these cells. We discovered that the autophagy process in CADASIL VSMCs is interrupted and, upon further investigation, found that this could be due to a dysfunction in fusion of the autophagosome and lysosome.

Next, we investigated the nutritional requirements in CADASIL VSMCs compared to control VSMCs. One of the most readily available forms of nutrients in myocytes is glucose. Hence, we examined glucose transporter expression in CADASIL VSMCs compared to control VSMCs. CADASIL VSMC glucose transporters were significantly down regulated and this down regulation had a negative functional effect such that CADASIL VSMCs were unable to take up glucose as avidly as control VSMCs. Adding insulin to the media only moderately increased glucose uptake in CADASIL VSMCs since Glut 4 was the only insulin-dependent glucose transporter that was down regulated (other glucose transporters were also down regulated, but they were insulin-independent).

VSMCs in CADASIL are frail and less resistant to stress. Based on this fact, we hypothesized that they may secret inflammatory cytokines and thereby affect their neighboring cells in paracrine fashion. Measuring inflammatory cytokine gene expression, we discovered that most of them were up regulated, which translated to an increased amount of protein synthesis. In this study, we have implicated inflammation

as a pathological driver in CADASIL. Dampening of this inflammatory response may slow the rate of VSMC loss and, consequently, disease progression.

LIST OF SCIENTIFIC PAPERS

I. Differences in proliferation rate between CADASIL and control vascular smooth muscle cells are related to increased TGFβ expression

II. Autophagy-lysosomal defect in human CADASIL vascular smooth muscle cells

III. Disrupted Glucose metabolism in Brain Myocytes in CADASIL

IV. CADASIL mutation in vascular smooth muscle cells cause high inflammatory response in CADASIL

CONTENTS

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
BMP	bone morphogenetic protein
CAA	cerebral amyloid angiopathy
CAD	coronary artery disease
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CBF	cerebral blood flow
CMA	chaperon-mediated autophagy
CMD	coronary microvasculature disease
CRD	cysteine rich domain
CRP2	cysteine-rich protein 2
CT	computed tomography
CVD	cardiovascular disease
EC	endothelial cell
ECM	extracellular matrix
EFGR	epidermal growth factor repeat
GLM	generalized linear model
GLUT	glucose transporter
GOM	granular osmiophilic material
haVSMC	human aortic vascular smooth muscle cell
HEK	human embryonic kidney
HtrA1	high temperature requirement protein A1
ICAM-1	Intercellular Adhesion Molecule 1
IL-1β	Interleukin-1-beta
IL-6	Interleukin-6
iPSC	induced pluripotent stem cells
Lamp2	lysosome-associated membrane protein 2
LC-MS/MS	liquid chromatography–mass spectrometry
LC3	microtubule-associated protein 1A/1B-light chain 3

LFA-1	lymphocyte function associated antigen 1
LTBP-1	latent TGFβ binding protein 1
MI	myocardial infarction
MRI	magnetic resonance imaging
N3ECD	NOTCH3 extracellular domain
N3ICD	NOTCH3 intracellular domain
NECD	NOTCH extracellular domain
NICD	NOTCH intracellular domain
NT	amino terminal domain
PAS	Periodic Acid Schiff
PDGF	platelet-derived growth factor
PET	positron emission tomography
PKR	protein kinase R
SGLT	sodium dependent active transporter
SMC	smooth muscle cell
SMMHC	smooth muscle myosin heavy chain
SVD	small vessel disease
TGFβ	transforming growth factor beta
TIMP3	tissue inhibitor of metalloproteinases 3
VSMC	vascular smooth muscle cell
VTN	vitronectin
WMHI	white matter hyper-intensities
α-SMA	α-smooth muscle actin

1 INTRODUCTION

1.1 OVERVIEW OF CADASIL

Cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy (CADASIL) is a genetic disease that is mapped to chromosome 19q13 [1, 2]. This disease was described as the presentation of ischemic strokes regardless of a history hypertension and hyperlipidemia, which made it unique when compared to the causes of stroke previously described [1, 2]. Although CADASIL genetics were only recently defined, this disease could have been discovered as early as 1955 when van Bogaert described a "Binswanger's disease with rapid course in two sisters" [2], but of course the methods to diagnose CADASIL did not exist at that time.

Clinical symptoms may vary between familial lines and based on the mutation site, but the core clinical manifestations consist of migraine with aura, subcortical ischemic attacks, mood swings or disturbance, apathy, and (perhaps the most noticeable) cognitive decline. CADASIL starts early in life, with migrainous symptoms manifesting as early as the teenage years. Disease progression is slow.

Because of its ambiguousness, the prevalence of this disease is hard to predict accurately; however, reports have suggested that the prevalence is 2-4 persons per 100,000 [3-5]. Although the majority of data regarding prevalence originates from Europe, non-European countries are increasingly reporting cases of CADASIL, suggesting that current prevalence estimations will increase with better diagnostic tools and a better understanding of the course of the disease. In Sweden, there are currently over 100 diagnosed CADASIL patients, with belief that an additional 300-400 cases exist that are either unreported or misdiagnosed. Currently there are over 150 NOTCH3 mutations identified that cause CADASIL [6].

1.2 CLINICAL INSIGHT

1.2.1 Blood Vessels

The human brain is supplied with blood via several major blood vessels, including as the vertebral, common, internal and external carotid arteries [7]. These major blood vessels for the circle of Willis and, therefrom, three cerebral arteries originate. These arteries further branch into smaller arteries that penetrate deep within the white matter. Most blood vessels in the brain run straight; however, upon entering the white matter they start to coil, branch off and loop [8]. While the gray matter is composed mainly of neuronal cell bodies, axons, dendrites, and glial cells, the white matter consists mostly of dense, myelinated axons [9]. Regardless of anatomical structure, almost all cerebral small vessels have a similar histological phenotype.

Cerebral blood vessels have a three-layer structure. The innermost layer, the *tunic intima*, is composed of a single layer of endothelial cells (ECs) held together by a tight cellular junction and an internal elastic lamina (Figure 1). In cardiovascular disease, damage to these ECs can cause infiltration of lymphocytes leading to plaque formation. The middle layer, the *tunica media*, is comprised of smooth muscle cells (SMCs), fibroblasts, and collagen fibers. The number of layers of SMCs depends on the size and location of the vessel. For example, the internal carotid arteries can have as many as 20 layers of SMCs, whereas smaller vessels may have only two or three layers of SMCs. The outermost layer, the *tunica adventitia* or *tunica externa,* consists mostly of collagen, fibroblasts, and associated cells [10].

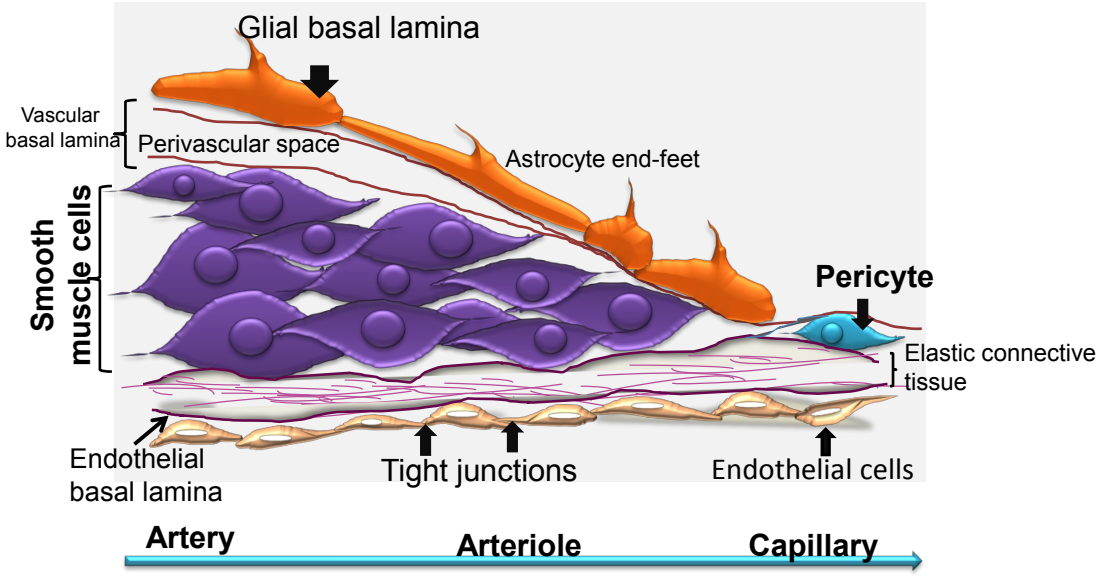

Figure 1: Illustrative representation of the three different layers of cerebral blood vessels.

1.2.2 Cerebral Small Vessel Disease (SVD)

The term small vessel disease (SVD) has come to encompass a collection of different diseases where small vessels are involved. Some of the diseases fall under the term SVD are coronary microvasculature disease (CMD), coronary artery disease (CAD), small vasculature vasculitis, and cerebral small vessel disease. Most investigation into small vessel disease has been focused on cerebral small vessel diseases, such as CADASIL. In fact, SVD has nearly become synonymous with vascular dementia and is responsible for about 45% of dementia cases [11, 12].

Cerebral blood flow plays a vital role in cognition and functional integrity of the brain and cerebral SVDs are connected to a disruption in such flow. There are also non-SVD conditions that are connected to disrupted cerebral blood flow, such as cardiac arrest, arrhythmias, cardiac failure hypotension, or stenosis. The exact mechanism of neuronal damage following low perfusion is unknown; however, chronic hypoxia, oxidative

stress, and inflammation have been identified as contributing factors for cognitive decline [7]. It is interesting that hypercapnia (a hypoxic condition that results in systemic vasodilation) does not increase cerebral blood flow but, on the contrary, reduces it in healthy subjects [13], suggesting that treatment of cerebral SVD requires a different approach than peripheral SVDs. It is reasonable to speculate that chronic hypoxia can, in turn, cause inflammatory cascades in the brain. Several pathological studies have identified elevated levels of various cytokines and adhesion molecules in the hypoxic brain. Additionally, post mortem studies have identified activated microglia and reactive astrocytes in the brain tissue of individuals who have suffered from hypoxia [7].

Given that SVDs are multifactorial disorders, no single intervention is sufficient for treating all types of SVD. CADASIL makes a good disease model to study SVD as the underlying cause of CADASIL is known to be a mutation in the *NOTCH3* gene and because other risk factors are not present – eliminating some of the complexities associated with studying other types of cerebral SVD. It is noteworthy that the same risk factors that apply to CAD and CMD can exacerbate CADASIL, albeit CADASIL patients most often lack any such risk factors.

1.2.3 Stroke

Stroke is a condition in which the blood flow to a portion of the brain is disrupted. This disruption in blood supply results in a localized area of necrosis in the surrounding tissue, which is called an infarct. In clinical terms, a stroke event in the brain is defined as (1) an acute focal brain dysfunction that lasts more than 24 hours, or (2) an acute focal brain dysfunction of any duration if computed tomography or magnetic resonance imaging shows an infarction or hemorrhage that can be correlated to the patient's symptoms [14]. Over the years, advances in stroke research have resulted in decreased mortality, but the incidence of stroke has steadily increased. Although patients who receive early treatment have good a chance of survival, their activities of daily living generally become greatly affected.

As of 2010, there were over 33 million reported cases of stroke survivors, representing a large increase since 1990 (about 18 million cases) [14]. This increase in could be (at least in part) due to an increase in the average lifespan, better diagnostic tools, and increased public awareness about the risk factors associated with stroke. Hypertension, atrial fibrillation, hypercholesterolemia, lifestyle choices (e.g., cigarette smoking, sedentary lifestyle, obesity, etc.), and carotid stenosis are among a growing list of such risk factors [15-17]. Although there are several stroke subtypes, hemorrhagic and ischemic stroke comprise the two major types.

1.2.4 Hemorrhagic stroke

In a hemorrhagic stroke event, the blood vessel ruptures and its contents are released into the brain parenchyma. While hemorrhagic stroke is less common than ischemic stroke, the former is more devastating and debilitating [18]. The risk factors associated with hemorrhagic stroke are the same as those associated with ischemic stroke, but the underlying cause of these two types of stroke differs greatly. The underlying causes of hemorrhagic stroke include hypertension (the most common cause) followed by cerebral amyloid angiopathy CAA, drug use, aneurysm, and previous ischemia.

1.2.5 Ischemic stroke

Conversely, in an ischemic stroke event, the vessel does not leak or rupture; it becomes blocked (or is narrowed to the extent that blood cannot flow freely). Moreover, the cause of ischemic stroke can be linked to hypercholesterolemia, hypertension, and cardiovascular disease (CVD). Based on current estimates, SVD is responsible for about 20% of all ischemic strokes [11, 12]. Ischemic stroke is the most common outcome of CADASIL and is also the most likely driver of pathogenesis. Although CADASIL patients lack the conventional risk factors for vascular disease, they are prone to transient ischemic attacks. These events occur later than the migraine attacks, with a mean age of 49 and a frequency as high as 85%. Most of these attacks are subcortical infarcts with subsequent lacunar syndrome (see *Lacunar stroke*). CADASIL patients may have 3-5 recurrent strokes over period of several years and this number increases with the addition of hypertension, smoking, hypercholesterolemia, and other conventional vascular risk factors.

These recurrent strokes lead to ataxic hemiparesis (which is a contralateral weakness), motor and/or sensory deficiency, dysarthria (difficulties formulating a coherent sentence or other linguistic difficulties), urinary dysfunction, and other stroke-related symptoms [19-21]. About 20% of patients suffer from mood disturbance, with apathy being the most common complaint. Other mood disturbances associated with CADASIL include depression with occasional manic episodes, which is easily mistaken for bipolar disease. It has been posited that the depression could originate from patients experiencing the feeling of becoming demented.

1.2.6 Lacunar stroke

In some cases, the necrotic tissue resulting from a stroke event is either removed by normal bodily processes or retracts, leaving an empty cavity (a lacuna) that is visible on diagnostic imaging tools as a black space (a lesion). These so-called lacunar strokes are the result of the blockage or rupture of a single, small, deep, penetrating vessel. The most common type of stroke in CADASIL is ischemic lacunar stroke.

There are four possible reasons proposed for lacunar infarcts: atheroma of parent artery or perforating arteriole, intrinsic vessel abnormality, and emboli. Based on the location of the lesion and resulting impairment, lacunar stroke could be further divided into pure motor hemiparesis, pure sensory stroke, ataxic hemiparesis, sensorimotor stroke and dysarthria syndrome [22]. Controversy has arisen to whether lacunar stroke is different than cortical ischemic stroke. In most cases, lacunar stroke are in deep penetrating vessels affecting the white matter and the cause of the stroke appears to be intrinsic cerebral small arteriolar abnormality while cerebral ischemic stroke involves emboli from larger vessels and the heart [23]. Among all the risk factors involved in stroke, hypertension emerges as one of the most important with regard to lacunar stroke. However, in the clinical setting, hypertension in lacunar patients is not correlated to severity of the outcome [24].

1.2.7 Diagnosis of stroke

The most common clinical features associated with stroke are neurological deficiencies. The most common symptoms are altered speech, ataxia, anosmia (loss of sense of smell), unilateral weakness and numbness, vertigo (sensation of spinning), headache, and diplopia (double vision). A trained physician in a clinical setting can easily identify these symptoms and, based on the neurological deficiency identified, one can have a good idea as to the location within the brain where damage has occurred.

After the initial assessment, a computed tomography (CT) scan can be performed to better assess the damage and to visualize the stroke. CT can be conducted either with or without contrast dye. The most frequently used form is non-contrast, also known as a dry CT scan. This technique is suitable for assessment of fresh hemorrhage, but its ability to detect ischemic stroke is poor. However, a magnetic resonance imaging (MRI) scan is can identify both old and recent hemorrhages, as well as detect brain ischemia [14]. Therefore, an MRI scan is the preferred tool to assess and visualize stroke.

1.3 CLINICAL SYMPTOMATOLOGY AND FINDINGS IN CADASIL

1.3.1 Migraine

About 20-40% of all CADASIL patients experience migraine with aura [2], the early onset of which has been associated with elevated levels of serum homocysteine [25]. Homocysteine is used as a marker of an elevated risk of endothelial damage, leading to inflammation and arteriosclerotic vascular disease [26]. These attacks can last from 20-30 minutes to a few hours. The severity of these attacks varies, with some patients experiencing visual and/or sensory aura while others experience confusion, fever, nausea and, in the most severe cases, coma [2].

Migraine triggering factors in CADASIL are the same as those of a classic migraine. The frequency with which CADASIL patients experience migraines varies greatly and is

correlated with familial lineage [27]. CADASIL diagnosis is challenging and misdiagnosis often occurs due to the fact that migraine in CADASIL closely mirrors a classic migraine, which is common among the general population. In fact, about 50% of CADASIL patients suffering from migraine present with the same clinical features as classic migraine episodes. As the disease progresses, additional symptoms begin to surface, including subcortical ischemic attacks, mood disturbance, and cognitive decline. Diagnosis is more commonly made when the patient begins to suffer from ischemic attacks.

1.3.2 Diagnostic imaging

MRI and CT are the two most common imaging modalities used for diagnosis of CADASIL. Nonetheless, MRI is not very useful at an early stage of CADASIL, as it lacks enough sensitivity to detect brain changes preceding the early clinical symptoms. There are three types of brain lesions observed using MRI and CT in CADASIL patients: (1) white matter hyper-intensities (WMHI) on MRI or white matter hypo-densities on CT, (2) lacunar infarcts – which is defined as occlusion of a deep penetrating artery, and (3) microbleeds.

The initial visible MRI changes are the WMHIs, and these are typically found in the anterior part of the temporal lobe and also in the periventricular region and deep white matter. The most affected cerebral areas are the frontal, parietal, and anterior temporal lobes, while the occipital lobe is less affected by CADASIL [28]. These types of WMHI are not only observed in CADASIL, but also other small vessel diseases. Because they are associated with dementia, research has been increasingly dedicated to understanding and predicting clinical outcomes based on their presence and prevalence. Reports suggest that the extent of WMHI visible on MRI (the more hyper-intensities observed), the worse the clinical outcome and severity of cognitive disturbance [29].

The most recent diagnostic imaging tool being used to study CADASIL is positron emission tomography (PET). Glucose metabolism malfunction has been reported in other dementia diseases and now there is evidence that glucose metabolism is altered in CADASIL as well. Tuominen et. al. were able to show a correlation between the reduced cerebral blood flow associated with CADASIL and glucose uptake. Although these initial observations were not statistically significant, additional studies suggest glucose uptake is altered in the CADASIL brain [30, 31].

1.4 CADASIL GENETICS

1.4.1 NOTCH signaling

NOTCH proteins are a family of type 1 transmembrane proteins involving in NOTCH signaling. In mammals, there are four NOTCH proteins, NOTCH1-4. In simple terms, NOTCH receptors contain a NOTCH extracellular domain (NECD) and a NOTCH intracellular domain (NICD) that are joined together by a single transmembrane

domain. Both NECD and NICD interact with various ligands. Thus, this family of protein receptors can be further subdivided based on their interacting ligands (e.g., Delta-like protein family and Jagged protein family) [32, 33].

These ligand-mediated transmembrane receptors are relatively simple in operation, but are key actors in developmental processes and diseases. NOTCH signaling is a highly conserved pathway and plays an active role in cell differentiation and apoptosis. All members of this family of proteins are similar in structure where the NECD contains a series of epidermal growth factor repeats (EFGRs). NOTCH signaling is initiated when NOTCH receptors on the cell surface engage ligands presented *in trans* on opposing cells.

Jagged ligands contain a cysteine rich domain (CRD) while Delta ligands lack this region, which is upstream of EGFRs. These ligands have both Delta-Serrate-LAG3 (DSL) and amino terminal domain (NT) that binds to EGF repeats 11-12 for activation of pathway. NT also has a phospholipid binding ability, suggesting that it may help anchor and stabilize the connection between the sending cell and receiving cell.

A negative regulatory region exists on NECD, which consists of an interacting heterodimeric portion of NOTCH and Lin127 NOTCH repeats. This region is able to shelter the cleavage site until a ligand has become bound to NECD. Upon activation, ADAM 10 makes the first cleavage at this site. The first cleavage is followed by a second cleavage, which is accomplished by ϒ-secretase. Once cleavage has occurred, the NICD travels to the nucleus where it interacts with DNA binding protein CBF-1 suppressor of Hairless-LAG1 and mastermind, which function as co-activators thereby initiating transcription machinery [32].

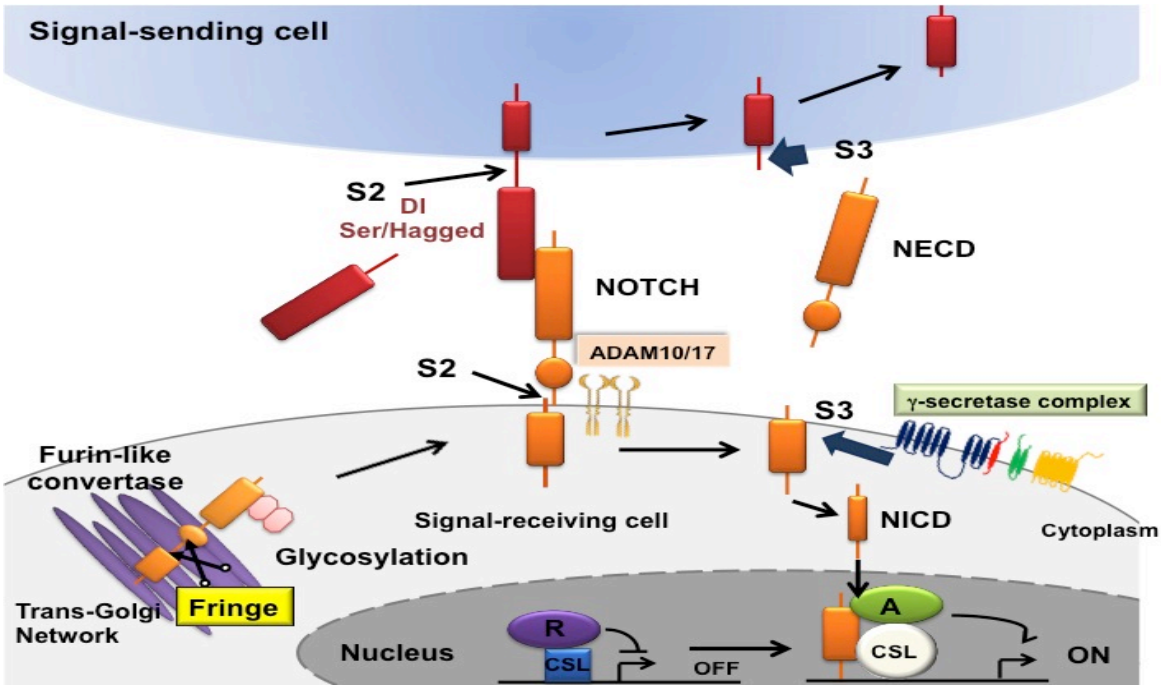

Figure 2: NOTCH cellular signaling depicting NOTCH receptor and its cleavage sites. Upon receiving signal, NOTCH is cleaved and NECD is released while NICD is further cleaved before entering the nucleus.

1.4.2 NOTCH3

Genetic risk factors of CADASIL have been mapped to chromosome 19q13 and many mutations on *NOTCH3* gene. This gene encodes for a single transmembrane protein, NOTCH3, which is composed of 2,321 amino acids, a NECD that has 34 EGFRs, and a NICD that is composed of seven ankyrin repeats [34]. Each EGFR has six cysteine residues. The mutation pattern in this region is essential for CADASIL pathogenesis.

NOTCH3 forms a heterodimer to its ligand receptor on the adjacent cell, which contains the ligands Jagged and Delta. Upon ligand activation, the NOTCH3 receptor undergoes a cleavage-dependent activation process. NOTCH3, like other NOTCH-family proteins, has three cleavage sites: S1, S2, and S3. S1 is activated during maturation while the S2 and S3 cleavage sites are ligand-binding dependent. S2 cleavage releases N3ECD, leading to S3 cleavage and the release of N3ICD. Upon cleavage, N3ICD, similar to other NICDs, translocates to the nucleus where it activates transcription factors CBF-1/RBPJκ.

1.5 CADASIL PATHOLOGY

1.5.1 Granular Osmiophilic Material (GOM)

While clinical symptoms and mutations vary between patients, the common pathological hallmark in all CADASIL patients is granular osmiophilic material (GOM). GOMs are detected by a staining technique known as Periodic Acid Schiff (PAS). GOMSs are defined as PAS positive material that measures about 1-2 μm in size [5, 35]. Gray and white matter studies in the CADASIL brain have shown that there are GOM deposits associated with meningeal vessel and perforating arteries. GOMs are exclusively extracellular deposits and are associated with NOTCH3 extracellular domains (N3ECDs) [36].

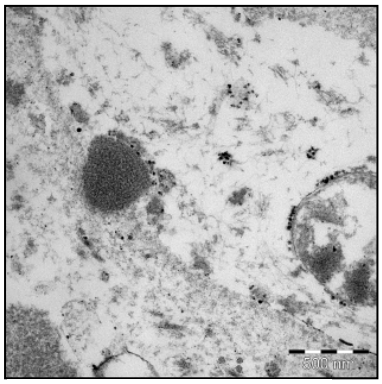

Figure 3: Electron micrograph picture of granular osmiophilic material (GOM) (photo: Docent Kjell Hultenby, KI).

GOM deposits have also been observed on arterial smooth muscle cells in skin, retina, muscle [37] and peripheral nerve [38] and have functioned as a golden standard for diagnosis of CADASIL via electron micrographic imaging. The exact composition of GOM is highly controversial, with conflicting reports. The only component that has been consistently verified by several groups is N3ECD [36, 39]. Recently, GOM depositions were studied in postmortem human brain tissue with R1031C mutation. The tunica media was targeted in brain arteries, resected by laser capture microdissection, and analyzed by mass spectrometry. Proteomic analysis data demonstrated that collagen 18 α1 and clusterin (also known as Apol) were prevalent in CADASIL samples compared to control samples, and presented as GOM components [40, 41].

Another group using nano liquid chromatography–mass spectrometry (LC-MS/MS) has analyzed NOTCH3 R90C transgenic mice and identified tissue inhibitor of metalloproteinases 3 (TIMP3) and vitronectin (VTN) proteins co-localized with N3ECD. Logically, as GOM is composed of N3ECD (among other proteins), it has been hypothesized that TIMP3 and VTN should also be associated with GOM. Proteomic analyses of vessels prepared from the brains of CADASIL patients supports the notion that TIMPS was enriched in GOM [42]. Thus far, GOM composition cannot be clearly delineated and whether its composition is static or is mutation/location dependent all remains undetermined.

1.5.2 Pathology of vessels in CADASIL

CADASIL causes narrowing of the lumen of small arteries. The deep penetrating and small arterioles are most commonly affected; however, the malfunction of blood vessels can be seen in medium-sized blood vessels as well [43]. Pathological findings have so far focused on the brain and central nervous system since the brain is the most vulnerable organ to blood flow regulation, but CADASIL pathology has been reported and observed in the small vessels of other organs as well, such as the kidney, eyes, liver, and heart [44].

There is evidence that the walls of the affected blood vessels become thickened and fibrotic in CADASIL due to deposition of extra cellular matrix (ECM) proteins, such as collagens [45], laminin [46], and fibronectin [47].

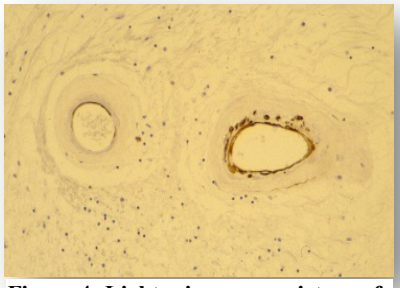

Figure 4: Light microscope picture of a vessel from a CADASIL patient showing fibrotic thickening.

Several pathological studies have provided evidence of reduced cerebral blood flow (CBF) in CADASIL [30, 48]. Morphological studies corroborate such evidence with findings of white matter arteriole stenosis, which in turn explains the impaired endothelium-dependent vasodilation in resistance arteries in CADASIL [49]. Another study by Henshall et al showed that the blood vessel integrity in the central nervous system is *NOTCH3*-dependent. It further suggested changes in *NOTCH3* gene expression compromise vascular integrity [50]. Therefore, the degeneration of VSMCs in microvessels and the impaired vasodilation of vascular cells can presumably be affected by the changes of CBF in CADASIL. Recently, we published a paper suggesting that TGFβ3 may be involved in ECM protein synthesis and thereby causative of one of the factors in fibrosis formation that disrupts the normal composition of vasculature in CADASIL [51]

The *tunica adventitia* is composed of collagen and, in the case of CADASIL, different types of collagen have different expression patterns depending of the type of blood vessel [52]. In leptomeningeal arteries, collagen-I was loosely packed and became

intensely stained compared to control vessels. This phenotype is also observed in small penetrating arteries. However, capillaries have the same collagen-I pattern of expression and phenotype in both CADASIL and control vessels. Collagen-III showed a similar staining pattern as collagen-I. The main difference was that more transmural staining extended into the *tunica media*. The most pronounced difference concerns the staining pattern of collagen-IV in CADASIL vessels. Leptomeningeal arteries showed large collagen-IV deposits in the *tunica media* and *tunica intima*. While the same phenotype was observed in small penetrating arteries, there were virtually no differences in collagen IV staining between CADASIL and control capillaries [52]. These types of fibrotic thickening cause restricted blood flow, which renders CADASIL patients more prone to strokes.

1.5.3 Endothelial Cells (ECs)

As previously mentioned, blood vessels consist of the *tunica intima*, *tunica media*, and *tunica adventitia* layers. While the *tunica intima* is not directly affected by CADASIL, some reports show that ECs have an abnormal morphology. In a study of CADASIL patient skin biopsy, Ruchoux et al have reported that ECs exhibited a "swollen" appearance that disrupted the tight junction functionality [53]. In a more recent study, Ling et al have generated vascular endothelial cells from a patient with CADASIL mutation by employing induced pluripotent stem cells (iPSCs) technique. They did not observe any abnormalities in these cells. However, it should be noted that only one patient was involved in the study [54].

1.5.4 Vascular Smooth Muscle Cells (VSMCs)

Because the *tunica media* is composed almost exclusively of VSMCs, it is the layer most affected by CADASIL. In CADASIL, the *tunica media* is significantly thinner when compared to a control vessel. The reason for such thinning is still a subject of debate with no conclusive evidence at hand. Some reports indicate that VSMCs with NOTCH3 mutation have a higher rate of apoptosis, while others found that the proliferation rate of VSMCs is lower than control and observed no signs of apoptosis [55, 56]. Nevertheless, different mutations in the *NOTCH3* gene may have varying affects the VSMC apoptosis and proliferation rates. In addition, reports suggest that mutant NOTCH3 may result in faulty VSMC conditions such as inappropriate entry into mitosis, irreversible arrest of the cell cycle, senescence, degeneration, or loss [57].

VSMCs are able to change phenotypes to adapt to their microenvironment via inflammatory factors, cell-to-cell interaction, growth factor, and other avenues [58, 59]. Upon assault and/or injury there is a release of platelet-derived growth factor (PDGF) followed by down regulation of α-smooth muscle actin (α-SMA), smooth muscle myosin heavy chain (SMMHC) and SM22-α [60]. Interestingly, PDGF receptor β is elevated in

CADASIL patient vessels and is associated with pericytes [61]. Nonetheless, NOTCH3 expression is not under direct control of PDGF [62].

Since the VSMC layer in the wall of CADASIL vessels is thinner than normal, it has been hypothesized that NOTCH3 may have an anti-apoptotic function. This is supported by a study using rats that constitutively express N3ICD, where VSMCs were resistant to Fas ligand induced apoptosis [63]. The role of NOTCH and CBF-1/RBP-Jk has also been investigated and it was found that their cooperation is essential in cellular homeostasis and that their dysregulation leads to diseases such as T-lymphoblastic leukemia and other forms of cancer [64]. Whether the aforementioned anti-apoptotic function of NOTCH3 is CBF-1/RBP-Jk dependent is still highly controversial [65, 66].

1.5.5 Pericytes

Pericytes have diverse functions and are seen mostly in capillaries. Their functions in central nervous system capillaries are maintenance of the blood brain barrier, gatekeeper for immune cell entry into the brain, and regulation of blood flow. These cells induce constriction of capillaries after an ischemic event, which results in the trapping of blood cells and prevention of microcirculatory reperfusion. Initially, this function was believed to be a task of arterioles; however, it was recently shown that capillaries are more efficient at regulating blood flow [67]. Because capillaries are also affected by CADASIL, Dziewulska et al have investigated morphological differences in pericytes in CADASIL brain and skin biopsies. They have reported a loss/reduced number of pericytes in capillary vessels as well as morphological changes, such as shrunken appearance with large cytoplasmic vacuoles [68].

Subsequent studies performed in mice have suggested that NOTCH3 aggregation and accumulation is the cause of pericyte pathology in CADASIL. The same group also reported a loss of pericytes that is correlated to age-related accumulation of NOTCH3. They confirmed the loss of pericytes in CADASIL and also linked pericyte loss to astrocyte end feed detachment, leakage of plasma protein, and reduced expression of endothelial adhesion protein (which is required for formation of cellular tight junctions). The most important finding of these studies is perhaps that they were able to show that these phenomena occur before the loss of VSMCs, suggesting that the disease initiates with loss of pericytes [69].

1.5.6 NOTCH3

The role of the *NOTCH3* gene is generally unknown and its expression pattern seems to be isolated to a few locations. *NOTCH3* expression has been reported in VSMCs [70]; however, other protein atlases and other resources suggest that *NOTCH3* could be expressed in locations other than VSMCs. Unlike a *NOTCH1* deletion which is lethal, *NOTCH3* knock out mice are fully viable and fertile [71]. This suggests that NOTCH3 protein is not important during the embryonic stage. Subsequent studies have,

however, illustrated that *NOTCH3* knock out mice have blood vessel enlargement, a thinner VSMC layer, and abnormally-shaped and -sized VSMCs. These mice exhibit vessel morphology that more closely resembles a venous, as opposed to arterial, vessel shape. Smoothlin, an arterial-specific marker that has been used to indicate the maturation of VSMCs, was markedly reduced in *NOTCH3* knock-out mice, suggesting that VSMC differentiation is altered in these mice [72].

1.5.7 Transforming Growth Factor Beta (TGFβ) and its role in CADASIL

Many different factors have been identified as regulating VSMC growth and proliferation. One of these factors of importance for us is transforming growth factor beta (TGFβ). TGFβ is a highly evolutionary conserved cytokine that is essential in embryogenesis and homeostasis in adult tissue. In the TGFβ family, there are three different TGFβ isoforms (TGFβ1, TGFβ2 and TGFβ3), bone morphogenetic proteins (BMP), and activins [73, 74]. TGFβ is a secretory cytokine, which is generated in dimeric form and then cleaved and secreted [75]. TGFβ tightly regulates VSMC differentiation via TGFβRI and TGFβRII. Both DeltaEF1 and myocardin were able to activate SM22α actin transcription factor via TGFβ activation, suggesting that these factors are important for differentiation and/or morphological appearance of smooth muscle cells [76, 77]. Growth arrest in VMSCs is also TGFβ dependent and occurs via P38MAPK and Smad3. It is noteworthy that in the same study, they also showed that inducible nitric oxide synthase and interleukin-6 were both down regulated [78]. TGFβ also plays a role in smooth muscle cell migration, which is regulated by cysteine-rich protein 2 (CRP2). CRP2 is thought to be exclusively expressed in VSMCs. Over-expression of TFGβ was able to upregulate CRP2 and, hence, inhibit VSMC migration [79].

The link between the NOTCH3 and TGFβ has been investigated in myocardial infarction (MI) and cardiac pathology. In a study where a mouse model was used post-MI, exogenous NOTCH3 expression attenuated the fibrotic formation in and around the area of assault and was able to downregulate TGFβ expression. These data suggest that NOTCH3 has an anti-fibrotic function, at least in heart [80]. TGFβ and its role in fibrosis have been documented in a variety of diseases; however, its role in fibrotic stenosis of vessels in CADASIL has not been elucidated.

The notion that TGFβ may be involved in SVD and CADASIL originate from a study where high temperature requirement protein A1 (HtrA1) was identified as an activator in the TGFβ pathway [81]. Subsequent studies further illustrated the role of TGFβ in CADASIL. In a study performed by Haffner et al, fibrillin-1 and fibronectin enrichment were not co-localized with N3ECD, while latent TGFβ binding protein 1 (LTBP-1) was shown to co-localize with N3ECD. The conclusion was drawn that since neither fibrillin-1 nor fibronectin is co-localized with N3ECD, their enrichment is most likely due to fibrosis and LTBP-1, which does localize with N3ECD and is suggestive of N3ECD aggregation as a pathological driver [47].

Using a more sophisticated method for vessel isolation and proteomic analysis by mass spectrometry, previously unknown proteins were identified in CADASIL. Among them, HtrA1 was enriched in CADASIL samples and further investigation into this protein showed an overlap between it and N3ECD. HtrA1 substrates such as vitronectin, clusterin, and elastin were all enriched in the CADASIL brain, while gene sequencing of HtrA1 failed to identify any mutations that would inhibit its cleaving ability [82]. These data suggest that HrtA1 has lost its function in CADASIL, but it remains to be explained how this loss of function has occurred.

It is important to note that there may be variation between the experiments and results as TGFβ expression and regulation is based on the local environment. Therefore, the data collected from both our lab and other labs could be misguiding. External factors can affect TGFβ expression (and thus impact VSMC differentiation, viability, and migration) such as media composition, cell density, and temperature [83]. Moreover, the regulation between ECs and VSMCs is tightly controlled. One of the regulatory markers for ECs is TGFβ. ECs can produce latent TGFβ upon interaction with SMCs and thereby control SMC differentiation and function [83].

1.5.8 Inflammation in CADASIL

Inflammation is an important process in defending the body against invading microorganisms, as well as recruiting proper cells (e.g., immune cells, fibronectin, etc.) and cytokines (e.g., IL-6, IL-12) to the injured site. However, this process itself can become pathological in cases of self-reactivity and enhanced inflammatory response in the brain. Ischemic stroke, which represents about 80% of all strokes, has high inflammatory elements [84, 85]. Upon assault, microglia and astrocytes become activated and they, in turn, start the production of inflammatory mediators [84, 86]. There are, however, other cells such as ECs and VSMCs that are able to produce the same cytokine. The role of the cytokines is to regulate cell proliferation, activation, and differentiation. There are several types of cytokines that will be released in response to injury. However, for the purpose of this thesis we will focus on Interleukin-6 (IL-6), Interleukin-1-beta (IL-1β), Intercellular Adhesion Molecule 1 (ICAM-1), and TGFβ [86].

IL-6 attracts T-lymphocytes to the site of injury and is produced mainly by microglia (69). IL-6 levels peak about 24 hours post-stroke and its major role is pyrogenic in nature [87]. There are currently no studies performed on IL-6 function in CADASIL VSMCs; however, neuropathological examination of postmortem CADASIL brains has suggested that there is elevated levels of IL-6 in association with ECs [88].

IL-1β is initially produced by activated microglia and, just like IL-6, has a pyrogenic function. Following microglia excretion of IL1β, the astrocyte and ECs start their own production of this cytokine. Through the use a feedback loop, IL1β is able to further activate microglia and astrocytes, leading to enhanced IL1β secretion. It can also cause edema, which enhances adhesion of leukocytes to the endothelium [86].

ICAM-1 is expressed on several cell types, including ECs and VSMCs. The functional role of ICAM-1 differs according to where it is expressed. On endothelial cells, its major function is facilitation of diapedesis, where Lymphocyte Function Associated Antigen 1 (LFA-1) on lymphocytes binds to ICAM and initiates transmigration [89]. It is conceivable that since N3ECD is accumulated extracellularly, and because it is a major component of GOM, CADASIL may have an inflammatory element that develops in response to these pathological hallmarks.

1.5.9 Glucose metabolism and CADASIL

PET scan results from several papers have shown a reduction in glucose uptake and/or metabolism in CADASIL brains, but the fundamental molecular mechanism behind such findings has not been studied [31]. There are other disease involving dementia, such as Alzheimer's disease (AD), where glucose metabolism and uptake has been better studied in mechanistic fashion.

Glucose is transported to the cell via two different transporters: (1) glucose transporter (GLUT), which is a facilitative sodium independent transporter, and (2) sodium dependent active transporter (SGLT). Throughout the body there are different forms of GLUT transporters, with GLUT1 being highly expressed in multiple types of cells including, brain epithelial cells, glial cells, blood tissue barriers, eye cells, peripheral nerve cells, placental cells. GLUT2 is more restricted, being found in astrocytes, liver cells, islets cells, and epithelial cells of the small intestines and kidneys. GLUT3 expression can be observed in neurons, testicular cells, placental cells, and brain endothelial cells. GLUT4 is different from the other glucose transporters as it is insulin-sensitive and its expression is more muscle-related. It is expressed in skeletal muscle cells, smooth muscle cells, and myocardial cells. It is also expressed in brown and white adipose neurons in the cerebellum and hippocampus [90].

Several studies have concluded that decades before clinical symptoms of AD appears in patients, there is a reduction of glucose metabolism in the brain. There are several working hypotheses that attempt to explain the altered glucose metabolism in AD. One such hypothesis is that neurons are less active due to mitochondrial malfunction and, hence, there is a decreased need for sugar. A second hypothesis is that neurons are more sensitive to damage and with death of these cells, the glucose requirement is decreased [91].

Nevertheless, there seems to be a correlation between glucose uptake and metabolism and neurodegenerative disorders. Whether the cerebral hypotrophy observed in CADASIL is the cause or effect of glucose malabsorption and metabolism remains to be seen.

1.5.10 Autophagy

There are generally three different ways endogenous proteins and particles can become degraded via lysosome. Degradation can be accomplished via chaperon-mediated autophagy (CMA), micro- or macroautophagy [92]. Macroautophagy is capable of degrading aggregations, and is therefore a rather interesting candidate for the degradation of aggregated NOTCH3 [93].

The process of macroautophagy can be summarized into five steps [94]. Once macroautophagy is stimulated, a phagophore is formed, which is an isolation of the membrane. After initiation, the phagophore undergoes elongation and proteins targeted for degradation are delivered by p62/SQSTM1. p62/SQSTM1 is capable of binding to LC3-II [95], an isoform of microtubule-associated protein 1A/1B-light chain 3 (LC3), which is bound to the phagophore, allowing for the incorporation of p62/SQSTM1 and the misfolded protein into the phagophore. A completed phagophore is called an autophagosome and it fuses with lysosomes, forming an autolysosome that is capable of degradation. Deficits in the autophagic-lysosomal pathway result in protein aggregation, the generation of toxic protein species, and accumulation of dysfunctional organelles. These are characteristics of many neurodegenerative disorders.

In neuronal development, autophagy has been observed to be detrimental as deletion of ubiquitous autophagy genes are always embryological lethal (as detected in knockout mice), with the exact function in neurons still under investigation. Initially, it was believed that the role of autophagy was to degrade proteins that were prone to aggregation, such as those seen in Huntington disease, but later it was shown that autophagy is also important in homeostasis and cell survival [96].

Autophagy has shown itself to be important in other neurodegenerative diseases such as AD. One of the hallmarks of this disease is the amyloid-beta (Aβ) formation and accumulation, which is degraded by the autophagy process, and increasing the rate of this process decreases the levels of Aβ. However, autophagosomes contains both amyloid precursor protein (APP) and Presenilin-1, which cleaves APP to form Aβ. This raises the question of whether Aβ is actually formed with the help of autophagosomes [97].

Although the list of neurodegenerative diseases where autophagy is involved is long, very little is known about autophagy process in CADASIL. To date, there are three reports that indicate involvement of autophagy in CADASIL. It the first report, Cognat et al investigated the protein kinase R (PKR), a pathway that is involved in apoptosis and cellular stress and can potentially activate the autophagy process. Using four postmortem CADASIL brain tissues and control brain tissue, they reported that phosphoPKR levels where higher in the CADASIL brain compared to control. However, using LC3 and ATG5 markers as indications of autophagy, they were not able to distinguish a different in levels between the control and CADASIL brain hence they concluded that autophagy is not a player in CADASIL pathology [98].

While Hase et al studied astrocytes, they realized that these cells in CADASIL have larger cell bodies and shortened or absent fibers compared to control brain. The CADASIL brain also exhibited a reduced number of astrocytes, which instigated the study of degradation in these cells. Using LC3 and p62/SQSTM1 as autophagy markers, the group investigated the role of autophagy and degradation of astrocytes in the CADASIL brain. Contrary to the report from Cognat et al, this group found elevated LC3 and p62/SQSTM1 in astrocytes. They concluded that the degradation machinery of these cells is associated with the autophagy pathway [99]. Given the findings from these studies, it is still unclear if this autophagy plays a role in the pathological events of CADASIL, or if it is a subsequent reaction to NOTCH3 aggregation and accumulation.

Our group was the first group to present findings that the autophagy pathway is disturbed in CADASIL VSMCs. We illustrated that lysosome-associated membrane protein 2 (Lamp2) a lysosomal marker was elevated in CADASIL VSMCs and this was correlated to an increase in the LC3/LC2 ratio. These data suggest that there is an increase in autophagosome formation and that the problem of NOTCH3 aggregation seems to be due to autophagosome inability to dock (fuse) with lysosomes when aggregated NOTCH3 should become degraded [100].

1.5.11 Animal models in CADASIL

One of the best ways to conduct research is by creating an animal model that mimic the human disease. In the case of CADASIL, this task has seen strenuous efforts with little success of mimicking CADASIL disease pathology.

Perhaps the most robust and extreme way to study CADASIL would be to knock out the *NOTCH3* gene, creating a *NOTCH3*$^{-/-}$ mouse model. This model is viable and fertile, meaning *NOTCH3* is neither essential for development nor for reproduction. Interestingly, this mice model showed none of the classical clinical phenotypes seen in CADASIL. There was clearly no *NOTCH3*$^{-/-}$ aggregation or N3ECD accumulation since the models lacked *NOTCH3*, but they also did not exhibit any white matter lesions, lacunar infarct, GOM formation or motor deficiencies. Upon histological analysis, the *NOTCH3*$^{-/-}$ mouse did, however, display asymmetric dilation and contraction of arteries derived from anterior part of the Circle of Willis. Additionally, the arteries of the brain resembled veins with a smaller and thinner smooth muscle cell layer. Interestingly, cerebral arteries in these mice were unable to respond well to pressure elevation but responded well to pharmacological agents that affected myogenic tone. These data suggest that NOTCH3 is important in autoregulation of cerebral blood flow and maintenance of hyper/hypo-perfusion [101].

Mouse models carrying varied CADASIL mutations have repeatedly been used, including mice carrying either the C455R or R1031C mutations in NOTCH3. The first description of C455R CADASIL mutation was discovered in a Colombian family in whom MRI abnormalities were more extensive. The R1031C mutation was also described in a Colombian pedigree [102].

C455R is located in the ligand-binding domain of the NOTCH3 receptor and has been shown to be involved in the early onset of stroke [102]. On the other hand, R1031C is located outside of ligand-binding domain of NOTCH3. The mice carrying these mutations develop GOM and other age-dependent phenotypes that resemble the human condition [40]. Using these animal models, many research groups have investigated pathological changes in CADASIL, performed principal testing of experimental drugs, or developed new therapeutic treatments for CADASIL.

Other attempts to generate mice via human full-length NOTCH with R90C and C428S mutations yielded conflicting results. Both of these models were able to produce GOM and N3ECD accumulation, but were void of white matter lesions, lacunar infarcts, and motor deficiencies. One caveat to generating these transgenic mice is that they exhibit 85% to 150% expression of endogenous murine NOTCH3.

Moving from mice to rats, CADASIL-like models carrying either full length *NOTCH3* WT or *NOTCH3* mutation R169C have been studied. These rat models demonstrated closer phenotypes to human CADASIL than current mice models, exhibiting both GOM deposits and N3ECD accumulation. However, these rats expressed 2-fold and 4-fold more NOTCH3 protein levels. While the rat that expressed a 4-fold increase of NOTCH3 exhibited white matter lesions, the rat that expressed only a 2-fold increase in NOTCH3 expression lacked white matter lesions. However, both of these rats lacked lacunar infarct and motor deficits, suggesting protein accumulation on its own does not cause CADASIL [43, 101].

1.5.12 Current treatment

CADASIL symptoms are exacerbated if a patient has other cardiovascular risk factors and treatment plans are based on the current treatment options for cardiovascular diseases.

Currently, there is no customized treatment for CADASIL and, therefore, the selected treatment regimen is entirely pragmatic. For the migraine, one should be very hesitant to use traditional triptan remedies as they work as vasoconstrictors. The recommended treatments are non-steroidal anti-inflammatory drugs (NSAIDs) for pain management, prophylactic β-blockers to regulate heart rate, and antiepileptic drugs.

Prophylactic treatment of ischemic attacks is much more difficult. Caution has been urged against the use anticoagulants, such as Coumadin, as these medications may increase the risk of intracranial hemorrhage. Anti-platelet drugs are used in these cases as they are deemed to be safer. Anti-hypertensive drugs are used if needed. Statin drugs, commonly prescribed for hypercholesterolemia, are currently used in CADASIL patients to reduce HMG CoA in the cholesterol pathway. These drugs have been proven to reduce low-density lipoprotein (LDL) levels; however, they have a large number of side effects [2].

There are currently 11 clinical trials either underway or completed with CADASIL as a main or side target. The most promising treatment being tested is donepezil, which has been used against dementia in AD. Donepezil is a reversible acetylcholinesterase inhibitor. In a clinical study testing donepezil, 168 patients with CADASIL were selected and 86 were treated with 10mg/day donepezil while 82 received placebo. 18 weeks post-study, there were no significant changes in the two groups, but there was improved cognitive function in the CADASIL group [103]. Other main targets in CADASIL drug development have been directed toward anti-platelet drugs, but recently there has been a shift in focus to *NOTCH3* mutation correction [104].

2 JOURNEY OF THIS THESIS

When we embarked on this PhD project four years ago, not much was known about the molecular pathogenesis of CADASIL, and most of the information at hand was case studies in clinical settings. The limited information about CADASIL was obtained by transfecting human embryonic kidney (HEK) 293 cells with NOTCH3 and NOTCH3 mutant containing plasmids.

Initially, we sought to transfect human aortic vascular smooth muscle cells (haVSMC) with different NOTCH3 mutations that we had constructed. The transfection became more daunting and time consuming than we expected. In our arsenal of tools, we also had immortalized umbilical, cerebral, and placental VSMCs taken from CADASIL patients with the C133R mutation. After a year of working on these cells and trying to transfect haVSMC simultaneously, we decided to shift our focus towards cerebral VSMCs with CADASIL mutation. We realized that although these cells were derived from CADASIL patients with the same mutation, they behaved differently under the same conditions. Therefore, we concluded that to be able to get the most physiological relevant data, we should continue our research with cerebral VSMCs obtained from control patients and CADASIL patients.

Throughout this journey, variations between the different VSMCs became apparent. We have learned that VSMCs obtained from small vessels in the brain behave differently than VSMCs from other types of vessels (e.g., aortic or placental) under the same conditions. This observation has opened a new area for further research – to study the differences between these cells and the cause of such differences. Because VSMCs obtained from brain and placenta exist in different environments, it is possible that their functions are different.

3 AIMS

The aims of this PhD thesis and work were to establish groundwork for clinical events in VSMCs containing CADASIL mutations.

Aim I

In the first paper, we studied the proliferation rate of CADASIL VSMCs with R133C NOTCH3 mutation. We hypothesized that TGFβ is involved in the lower proliferation rate observed in CADASIL VSMCs.

Aim II

The role of autophagy was investigated in this study. We tested the hypothesis that impaired autophagy inhibited the proper degradation of aggregated NOTCH3 in CADASIL.

Aim III

In this study, the metabolic deficiencies in CADASIL VSMCs were examined. Our hypothesis was that glucose transporters are down regulated in CADASIL VSMCs.

Aim IV

The question of whether inflammation is apparent in CADASIL VSMCs was investigated in this study.

4 MATERIAL AND METHODS

4.1 ETHICAL CONSIDERATIONS

Studies involving human subjects carried out as part of this thesis were conducted under proper ethical permissions. Ethical permissions were obtained from the Ethical Board of the Hospital District of Varsinais-Suomi, the Ethical Committee of Turku University Hospital, the National Authority for Medicolegal Affairs in Finland, the Regional Ethical Review Board of Stockholm, the local research ethics committee of the Newcastle Brain Tissue Resource (NBTR), or the Research Ethics Committee of the South Huddinge University Hospital. Human subjects gave informed consent, when required.

4.2 ESTABLISHMENT OF CELL LINES AND CELL CULTURE

Multiple vascular smooth muscle cell (VSMC) lines were used for experimentation in Papers 1-4 of this these. All VSMC lines used for the experiments performed in this thesis originated from genetically verified CADASIL patients with the R133C mutation ($VSMC^{CADASIL}$) and control subjects ($VSMC^{WT}$). Genetic verification was done by gene sequencing [105]. VSMC lines were established from small blood vessels obtained postpartum from pregnancies, in which either of the parents was diagnosed with CADASIL (human umbilical cord arterial VSMC, and human placental VSMC), or from post mortem subarachnoid branches of cerebral arteries (human cerebral arterial) [105-107], or from human foreskin fibroblasts [108].

Umbilical, placental, and cerebral VSMCs and human foreskin fibroblasts [108] were cultured in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F-12 with GlutaMAX, supplemented with 10% heat-inactivated fetal bovine serum and Penicillin-Streptomycin (100U/mL/ - 100µg/mL) at room temperature. Incubation of cell lines was performed in an incubator with 5% CO2 at 37°C. Cells were passaged every 2 days with a passage number of 18-28. To partially immortalize the cell lines, they were transduced with a human papilloma virus construct p6/p7 in early passage (p3–p5) [105]. Transduced cells were selected by culturing the cells in the presence of G418 (400 µg/mL) for a 10-day period.

4.3 HUMAN SUBJECTS

Where human subjects have been used for this thesis, the mean age of CADASIL subjects were matched to the control subjects. Available case notes and radiological reports indicated that CADASIL subjects showed extensive white matter changes consistent with small vessel disease of the brain and met the minimum criteria for cognitive impairment used in our post-stroke survivors study [109]. CADASIL diagnosis was confirmed by the presence of *NOTCH3* gene mutations or the presence of GOM in

arteries within skin biopsies [110]. None of the controls had neurological or pathological evidence for cerebrovascular disease or neurodegenerative disorder.

The brain tissues from CADASIL subjects (frontal and occipital lobes) and aged-matched controls were from the NBTR, Newcastle University, Campus for Ageing and Vitality, and the Swedish Brain Bank (Stockholm, KI).

4.4 STAINING PROCEDURES

4.4.1 Primary Staining

VSMCs were fixed with 4% paraformaldehyde (for 10 min at room temperature, and permeabilized using 0.3% Triton X-100 in PBS for 20 min. at room temperature. Cells were then washed with PBS and blocked using either 3% normal goat serum or 1% bovine serum albumin (BSA) diluted in PBS for 30 min. at room temperature.

4.4.2 Double Staining

VSMCs were washed, fixed, and incubated as described above (*see Primary Staining*). Cells were then incubated overnight with primary antibodies; N3ICD and KDEL, as an endoplasmic reticulum (ER) marker at 4°C. The following day, the samples were washed 3 times with PBS and incubated for 1h with the secondary antibodies.

4.4.3 Triple Staining

For co-localization of NOTCH3 with lysosomal and autophagy markers, before and after treatment, triple immunostaining was also performed. The cells were treated with CQ for 24 h. The staining procedure was done as described above (*see Other Treatments*). The antibodies used for triple staining were: anti-LC3, anti-Lamp2, and anti-NOTCH3 followed by incubation with secondary antibodies.

4.4.4 Immunofluorescence Staining

Immunofluorescence staining was performed on 5 µm sections of formalin-fixed paraffin-embedded (FFPE) frontal cortex of post-mortem brains. The sections were de-paraffinized and hydrated through xylene and graded alcohol series. The sections were autoclaved with antigen retrieval buffer for 30 min at 110°C. When room temperature was reached, sections were washed with water for 5 min. and then in Tris-Buffered Saline (TBS) + 0.05 % Tween® 20 (TBS-T). The sections were blocked with Background Punisher for 10 min. at room temperature, followed by washing and incubation with primary antibodies.

After washing, sections were incubated (1h at RT) with appropriate secondary antibodies. Sections were again washed in TBS-T (3 × 10 min) and then incubated with or without Sudan Black B, for 5 min. at room temperature to reduce auto-fluorescence. Sections were washed again in TBS-T and mounted with DAPI Vectashield Hard Set. Sections from controls and patients were also incubated without primary antibody and used as a negative control.

4.5 OTHER TREATMENTS

4.5.1 CFSE Labeling

Carboxyfluorescein succinimidyl ester (CFSE) is a membrane-permeant fluorescein-based dye that can be used to track cell division due to the progressive halving of the fluorescence intensity of the dye in cells after each division. After cleavage of the acetate groups by the cell esterases, the molecule becomes fluorescent and cell impermeant. During cell division, CFSE is partitioned equally among the daughter cells. That property is essential for visualization of cell proliferation where the intensity of CFSE fluorescence is a function of the concentration of CFSE and the duration of the staining. The dye has been used to monitor the proliferation of many other cell types, including smooth muscle cells [111].

CellTrace CFSE was used to enable tracking of VSMC proliferation. A 5 mM stock solution was prepared according to the manufacturer's protocol and kept at -80°C until used. VSMCs^{CADASIL}/^{WT} were grown to 90% confluence and harvested after washing twice with PBS. The collected cells were centrifuged for 5 min at 600 × g and the supernatant was removed followed by re-suspension of the cells in 1 ml pre-warmed PBS. One population of cells were labelled with 1 μM CFSE for 10 min at 37°C. The cells were then centrifuged for 5 min at 600 × g and the supernatant was replaced with 1 ml fresh medium. Labeled cells were counted by Trypan-blue using a hemocytometer, and equal numbers of CFSE-labeled cells were seeded on 24-well plates and incubated for either 3 or 7 days at 37°C.

Unlabeled cells were used as negative controls. For the loading control of CFSE, the cells were labeled with CFSE upon running through the flow cytometer in parallel with other samples. FL1 was used to detect VSMCs loaded with CFSE. Flow cytometry was performed using a FACSCalibur™ Cytometry. Approximately, 5,000-20,000 events were collected for each analysis. For direct VSMC culture, the CFSE- labeled VSMCs^{CADASIL}/^{WT} were seeded in 24-well plates and cultured with an equal number of the unloaded VSMCs$^{CADASIL/WT}$ on the same side for 3 and 7 days, harvested, and prepared for flow cytometry as described above.

For Paper II, VSMCs were treated with chloroquine (CQ) diluted in complete medium at variant time points. CQ inhibits lysosome protein degradation by increasing lysosome pH, which inhibits its proteolytic enzymes and causes the accumulation of autophagic

vacuoles [112, 113]. CQ concentrations were based on values presented by a previous report [114].

4.5.2 NBDG Glucose Uptake Assay

Cells were incubated and grown in DMEM (*see Establishment of Cell Lines and Cell Culture*) without the additional FBS and glucose for 24 h to a monolayer confluence. Later, the medium was removed and cells were washed 3 × with PBS Krebs ringer phosphate buffer with 50 mM glucose and 200 µM 2-NBDG (2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) was added to the cell layer. Cells were incubated with and without 50 mM insulin for 30 min. The cells were harvested and the 2-NBDG uptake was measured by flow cytometry using a FACSCalibur™ Cytometry.

4.6 WESTERN IMMUNOBLOTTING

Western blotting was used in Paper I, II, III and IV for protein measurement and expression.

Total protein extracts (10-25 µg) were mixed with 2 × SDS sample buffer, boiled for 5 min and loaded onto NuPAGE 4-12% or 12% Bis-Tris using NuPAGE MES or MOPS SDS Running Buffer. Gels were blotted with the wet tank transfer system using Amersham Protran 0.45 µm Nitrocellulose membrane and transfer buffer. The membranes were blocked using 5% milk in TBS-T. After two TBS-T washing steps, the membranes were incubated ON at RT or 4°C with the primary antibodies diluted in 5% milk and 0.2 % sodium azide.

The membranes were washed with TBS-T and incubated with specific secondary antibodies, anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase (HRP) for 1 h, at RT.

For Paper III, $VSMCs^{CADASIL/WT}$ were treated as described above prior to buffering, except that they were grown to 80% confluence. Laemmli sample buffer was added to the samples. 2 × LDS sample buffer and used for SDS-PAGE and immunoblotting as previously described [115].

4.7 ANTIBODIES

4.7.1 Transforming Growth Factor-beta (TGFβ) Antibody

TGFβ Pan-specific neutralizing antibody was derived from porcine platelet-derived TGFβ1 and 2, which detects all isoforms of TGFβ receptors. VSMCs were seeded at 5 × 10^4 cells/mL in 24 well plates and cultured overnight. Later, the medium was changed and cells were incubated in the presence or absence of 12 µg/ml TGFβ-neutralizing antibody for 24 h.

To examine the effect of TGFβ produced by placental and cerebral VSMCs$^{CADASIL/WT}$ on endothelial cell proliferation rates, the placental and cerebral VSMCs$^{CADASIL/WT}$ and endothelial cells were co-cultured in a non-contacting co-culture transwell system (Pore size 0.4 μm). Endothelial cells were plated at 5 ×10^4 cells/mL in 6- or 24-well plates. Placental and cerebral VSMCs were seeded at 5 × 10^4 cells/mL onto the membrane of transwell cell culture inserts and allowed to grow overnight. After 24 h, the transwell insert membrane containing the VSMCs was placed into plates containing endothelial cells. VSMCs were treated with a TGFβ-neutralizing antibody (12 μg/ml).

4.7.2 Lamp2 Antibody

To examine the co-localization of NOTCH3 with lysosomes, in Paper II VSMCs were stained with mouse Lamp2 antibody to allow visualization the lysosomes. The cells were then co-stained with N3ICD antibody to allow visualization of NOTCH3. VSMCs were exposed to CQ and incubated for different incubation times (0, 1, 4, and 24 h). We have only used NOTCH3 antibodies that recognized the C terminus of N3ICD in study II.

4.7.3 p62/SQSTM1 Antibody

For co-localization of NOTCH3 with autophagy marker, VSMCs were stained with anti-p62/SQSTM1 antibody, and N3ICD.

4.8 QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (QRT-PCR)

qRT-PCR was used to investigate genes related to proliferation and the cell cycle in placental and cerebral VSMCs. VSMCs$^{CADASIL/WT}$ were grown overnight in a 6-well plate chamber with confluence of 100.000 cells. The following day, the cells were lysed with RIPA buffer and RNA was isolated from VSMCs$^{CADASIL/WT}$ using RNeasy Mini kit. The quality of isolated RNA was determined to have a RIN-value (RNA Integrity Number) of 10. cDNA was synthesized using using Taqman gene expression master mix and SuperScript VILO cDNA Synthesis kit according to the manufacturer's protocol.

qRT-PCR was performed using either (1) the TaqMan Array targeting human cyclins and cell cycle regulation or (2) costume format TaqMan fast plate. The TaqMan Array was used to examine whether such genes are disrupted in CADASIL. The panel contains probes targeting cyclins and cell cycle regulation associated genes, genes encoding members of the TGFβ superfamily of ligands, and four endogenous control genes.

The TaqMan Array Human TGFβ pathway plate was used for co-culture studies. All TaqMan Probe qRT-PCR reactions were performed in triplicate in 1× TaqMan Fast Advanced Master Mix with 30 ng of cDNA. qRT-PCR was performed on a 7500 Fast Real-Time PCR System.

4.9 IMAGING

4.9.1 Immunoflourescent Imaging

All immunofluorescent imaging was performed using a laser scanning confocal microscope (LSM 510 META, ZEISS, or core Facility Bionut Microscopy), and images were acquired using the same settings (laser intensity, detector gain and amplifier offset). Also, the tissues fluorescence staining was recorded sequentially in separate channels with Plan-Apochromate 20 × (NA, 0.8), 40 × (NA, 1.2) oil and 100 × (NA, 1.45) oil objectives. Image processing was performed with the included ZEN software. Cell quantifications were performed with Neurolucida. Fluorescence intensity was measured with the ImageJ 1.383 software.

4.9.2 Positron Emission Tomography (PET) and Magnetic Resonance (MRI) Imaging

Ten CADASIL patients (4 females, 6 males) with a confirmed R133C mutation on *NOTCH3* were used as study subjects. The patients represented 6 families and had a mean age of 33.7 ± 5.2, with a range of 24 to 41 years. Control subjects were comprised of healthy family members of the study subjects who were confirmed to be lacking the R133C mutation. Controls were assessed to have normal T1w/T2w MRIs and also had no history of neurological disorder [30].

Neurological examinations and PET scans of study subjects were performed on the same day. Cognitive status was assessed using the Mini-Mental State Examination (MMSE) [116]. PET scanning was performed using a GE Advance PET scanner in the 2-dimensional scanning mode. The axial and transaxial spatial resolution of the reconstructed images was approximately 5-mm full-width at half maximum. 2-[18F]-fluoro-2-deoxy-D-glucose ([18F] FDG) was synthesized as described earlier [117, 118]. The radiochemical purity exceeded 95%; 3.7 MBq/kg was injected intravenously and a dynamic scan for 55 minutes was performed. During the PET study, arterial blood samples were drawn from the radial artery.

Within one month of the examination and PET scan, MRI was performed. Study subjects underwent a 1.5 T brain MRI and 3-dimensiona T1 weighted sequence and T2 weighed sequence were acquired.

PET and MRI images were matched using a surface-fitting method, then re-sliced using trilinear interpolation. Circular regions of interest (ROI) were drawn manually on individual MRI images on the frontal and occipital white matter periventricularly, on the frontal, temporal, and sensorimotor cortices, and on the hippocampus, putamen, and cerebellum. The ROIs were then copied onto rCMRgluc images. The rCMRgluc values were calculated in mol/mL per minute using the graphical analysis method [30].

Statistical comparison of values between groups was measured by one-way ANOVA followed by Bonferroni's post-hoc test. Student *t*-test was used for two-group comparisons. p-values < 0.05 were considered significant. The result three independent biological replicates expressed as mean ± S.E.M.

Un-adjusted bivariate comparisons of the PET measures between the CADASIL and control groups were performed using independent samples *t* test. We then applied generalized linear models (GLM) to adjust these comparisons for age and sex as potential covariates in the models. Also, multivariate GLMs were used to explore differences in PET measures between males and females and how they interacted with the effect of aging on the cerebral metabolic rates of glucose in each region of interest. In each GLM, the $rCMR_{gluc}$ of each region was defined as the dependent variable, whereas age, sex, and age-sex interaction were the independent variables.

All univariate and multivariate analyses were performed using *IBM SPSS Statistics* software (version 23.0). A two-tailed *p*-value of < 0.05 was considered as the threshold for statistically significant differences or associations in all analyses.

5 RESULTS

5.1 STUDY I

The most apparent pathological marker in CADASIL is the fibrotic thickening of small vessels in the white matter region of the brain. The cause for this stenosis is currently under debate. It is clear that these vessels have at some point during the disease progression lost their VSMCs or that VSMCs have been replaced by fibrosis, making the *tunica media* layer thinner compared to a control blood vessels. There has been discussion that VSMCs in CADASIL are more prone to apoptosis and, hence, the thinning of *tunica media* occurs because of their higher apoptotic rate. The suggestion is that once the VSMCs have under gone apoptosis, they are replaced by fibroblasts, which results in the manifestation of fibrosis [63]. Our group has previously shown that the CADASIL VSMCs have a lower proliferative rate compared to control [56].

We further probed the cause of the low proliferation rate of CADASIL VSMCs by focusing on cell cycle pathways and TGFβ3. This finding was in accordance with other groups who suggested that TGFβ might be involved in CADASIL disease [47, 81]. TGFβ3 became the target as it has shown to be involved in fibrosis, VSMC function, and remodeling of the extracellular matrix [119, 120]. We noticed that CADASIL VSMC cell media is able to alter the proliferative rate of other cells once they are exposed to such media. Several cell lines such as EC, control VSMC, fibroblast and human aortic VSMC were affected by the CADASIL VSMC growth media supernatant. This phenomenon was rescued by adding neutralizing anti TGFβ3 antibody, suggesting that CADASIL VSMCs secrete TGFβ3, which inhibits its own proliferative potential as well as that of its neighboring cells. In this study, we improved our understanding of cell proliferation and implicated TGFβ as one of the players that regulate cell proliferation [51].

5.2 STUDY II

Studies confirm that one of the first events is CADASIL is the aggregation of NOTCH3. This aggregation can be found in in GOM as well as on the surface of VSMCs [39, 121]. The accumulation of this protein and the lack of its degradation was the subject of investigation in this study. Autophagy is one of the key degradation pathways in protein aggregation and we hypothesized that this pathway is affected in CADASIL. To further corroborate other studies, we performed a Thioflavin-S staining to illustrate protein accumulation. This staining is used widely as a screening tool for protein aggregation.

Next, we measured LC3 expression in CADASIL VSMCs compared to control VSMCs. LC3, an autophagosome marker, was significantly higher in CADASIL VSMCs, suggesting that there is a disruption in the autophagy pathway. This finding led us to analyze the fusion of the autophagosome and lysosome. Upon treatment with chloroquine, a lysosomal inhibitor, the degree of LC3 levels increased in control VSMCs while it remained steady in CADASIL VSMCs. These results suggested to us that the autophagy pathway is important in the clearance of aggregated NOTCH3 [100].

5.3 STUDY III

Metabolic investigation in CADASIL has been limited to PET images with the consensus being that glucose uptake is lower in the CADASIL brain. Some of these studies have attributed lower glucose uptake to impaired cerebral blood flow [30, 122]. Using CVD as inspiration, we revisited some of the PET scan data and, in line with CVD, observed that age and sex factors were associated with CADASIL disease progression.

Next, we sought to investigate the glucose uptake on cellular level in CADASIL VSMCs. Initial experiments in glucose uptake gene expression suggested that several glucose transporters had a lower expression level in CADASIL VSMCs compared to control VSMCs. We further investigated the functional effect of lower glucose uptake expression. As a baseline, CADASIL VSMCs had lower glucose uptake relative to control VSMCs. To increase this uptake, we used insulin to activate glucose uptake of Glut4. Although using insulin did increase glucose uptake in CADASIL VSMCs, it did not completely increase the glucose uptake to control level. This is most likely due to other glucose transporters, such as Glut2 and 3, which were also down regulated in CADASIL VSMCs being insulin-independent.

Here, we have demonstrated that although cerebral blood flow is one reason for lower glucose uptake in the CADASIL brain, lower glucose uptake is also observed in VSMCs. The question that remains is whether an increase glucose uptake in CADASIL VSMCs would make these cells more viable and, in turn, reduce the number of VSMCs in vessels, as it is a reported in CADASIL.

5.4 STUDY IV

In many neurodegenerative diseases, inflammation plays an active role. However, the role of inflammation and its activity has so far not been described or investigated in CADASIL. The VSMCs containing CADASIL mutation behave differently and their survival condition and proliferation differed compared to control VSMCs. Having the data from paper I, II, and III, and considering that inflammatory response is a major contributing factor to many vascular events, we sought to investigate whether mutated CADASIL VSMCs are able to invoke an immune response.

We initiated the study by reviewing different expression levels of genes that has been shown to be involved in inflammatory responses. The first observation by qRT-PCR technique showed high gene expression of inflammatory genes in CADASIL VSMCs compared to control. Among these genes, we focused on IL-6 and ICAM-1. Using western blot analysis, we could confirm that the observed high level of IL-6 and ICAM-1 gene expressions was similar to the protein level. When we co-cultured CADASIL VSMCs with an endothelial cell line, we observed that mutated VSMCs affected ECs by inducing a higher IL-12R expression suggesting that cytokines secreted by CADASIL VSMCs have an effect on the neighboring cells. Preliminary results of stained post mortem brain

tissues from CADASIL patients demonstrated highly activated microglial cells. Of note, there were nearly no indications of microglial positivity in control tissues. Preliminary data from immunohistochemical staining of activated microglial cells in CADASIL brain tissues suggest more general inflammatory responses than the local one generated by VSMCs. Even though this study is in its infancy, it is providing significant information of possible inflammatory effect involved in CADASIL pathology. Knowing that inflammation may affect the VSMCs in the brain, and there is a possibility of other inflammatory sources in the CADASIL brain, it can provide a novel disease targeting for dampening this inflammatory process thereby slowing down the disease progression.

6 DISCUSSION AND WAY FORWARD

Since the discovery that a mutation on the *NOTCH3* gene causes CADASIL in 1996, great strides have been made in CADASIL research but much more work needs to be done to fully understand the disease and its progression. The initial work on CADASIL was performed on cells that are easily transfected (e.g., HEK293 cells). These early studies shed light on issues such as signal transduction, protein aggregation, and cellular response. They also laid the groundwork for more advance and complicated animal models as well as more in-depth studies of CADASIL. There are many studies characterizing new CADASIL mutations, in addition to reports that constantly identify new families with CADASIL, co-morbidity with other diseases, and involvement of organs other than the brain.

ECG studies have indicated that CADASIL patients also have cardiac pathology, with 25% of CADASIL patients having an abnormal Q-wave or recorded MI [123, 124]. While it is hard to concretely claim that CADASIL affects the microcirculatory system of the heart, it is conceivable that CADASIL affects more than merely the brain microcirculatory system. Other studies have implicated the kidneys and ocular system as potential organs that can be affected. In the kidneys, evidence suggests that there is NOTCH3 aggregation/accumulation and GOM in the perivascular space, as well as stenosis of renal arteries. However, there is currently no evidence that kidney function has been affected [125, 126]. Large-scale studies are needed to conclusively rule out CADASIL as having no effect on kidney function.

CADASIL appears to affect the ocular vascular system as well. In a recent study, Nelis et al used optical coherence tomography angiography to show that CADASIL patients have a lower vessel density of the deep retinal plexus [127]. Previously, others have demonstrated stenosis of the central retinal artery and its branches, GOM deposition perivascularly, and nerve fiber loss. However, to date, there are no reports that CADASIL causes loss of vision [128, 129].

Considering that many organs are affected by CADASIL, there is a consensus within the CADASIL field indicating that aggregation of NOTCH3 is the cause for major pathology seen in this disease. But no direct link has been made between the angiopathy observed in CADASIL and NOTCH3 aggregation and accumulation. Several groups have tried to study the effect of NOTCH3 aggregation on vessels by inducing an immune response to clear out the aggregates, or by using antibodies to activate NOTCH3 signaling. Activation of NOTCH pathway is based on its cleavage. For this reason, Li et al designed a study where they were able to activate and inhibit NOTCH3 signaling in HEK293T cells with the help of antibodies generated in their group. Their work was focused on NOTCH3 signaling due to its role in cancer development and progression, especially in non-small cell lung cancer and ovarian cancer [130].

Due to the mural cell (VSMCs and pericytes) loss observed in CADASIL, later work used these antibodies to implicate NOTCH3 signaling in CADASIL disease. A mouse model was generated with a C455R mutation since this mutation is on the ligand-binding domain of NOTCH3 and is thought to disrupt the signaling greatly. It is also evident that human patients with this mutation have a more aggressive clinical manifestation of CADASIL [102]. Additionally, knock out mice lacking the *NOTCH3* gene were generated for comparison purposes. Although the knock out mice did not show any signs of stroke or changes in the white matter, they did exhibit loss of mural cells. The C455R mice presented GOM formation but, like the knock out mice, they also did not exhibit stroke or white matter changes in the brain. Adding agonist NOTCH3 antibody to C455R mice rescued signaling disruption while inhibiting the loss of mural cells. These data suggest that, in CADASIL, the signaling pathway disruption due to mutation of NOTCH3 is an important pathological driver and could be rescued via agonist NOTCH3 antibody [131]. This research raised the question of whether NOTCH3 aggregation is the causative factor in mural cell loss or if it is a byproduct of the disease. It also suggested that a loss of signal transduction in mural cells could be the underlying cause for the loss of such cells.

To further investigate the possibility that NOTCH3 aggregation is pathology causing, Ghezali et al studied whether passive immunization with N3ECD would improve CADASIL outcomes. Using NOTCH3 R169C mouse model, this study confirmed previous findings that N3ECD accumulates over time and that these animals exhibit cerebrovascular dysfunction with white matter lesions. Using an antibody directed towards the ECD of NOTCH3 (5E1), they injected the mice once a week for 20 weeks. They further investigated GOM and N3ECD deposition perivascularly, and concluded that there was no significant reduction in deposition between immunized mice and control mice. The recruitment of microglia cells around the vessel was not different between the E51 treated mice and control mice, and immunization had no effect on white matter lesions between these groups [132]. The major finding of this research was that there is improvement to myogenic tone in the 5E1 treated NOTCH3 R169C mouse model, suggesting that improving myogenic tone will not alleviate the white matter lesions observed in CADASIL.

In our initial work, we implicated TGFβ as one of the players involved in CADASIL pathology. We hypothesized that TGFβ secretion by VSMCs induces fibrosis as well as degeneration of VSMCs in the vessel. Initiating treatment in our cells with neutralizing TGFβ antibody restored the proliferative rate of CADASIL VSMCs compared to control VSMCs, raising the interest of neutralizing TGFβ antibody as a therapeutic means. In this study, we used a cell line that was established from one human with one CADASIL mutation, namely R133C. For that reason, the observed effect of NOTCH3 mutation on TGFβ could potentially be a cell line-specific or mutation-specific effect. It would be interesting to generate more cell lines with different mutations to test the effect those mutations have on TGFβ. Our study focused on TGFβ3, a subtype of TGFβ that is involved in fibrosis. This could be to our advantage as the neutralizing antibody specific for TGFβ3 could be used to inhibit fibrosis in CADASIL and increase VSMC

proliferative rate. To test the beneficial effect of neutralizing antibody specific for TGFβ3, we would need a CADASIL animal model where we could measure fibrotic thinking of brain blood vessels and disease progression. The major caveat with this therapy would be the off target affects this antibody would have and this therapy could potentially disrupt the normal philological function of TGFβ systemically.

In our next project, we demonstrated that autophagy was involved in CADASIL disease progression and accumulation on NOTCH3 aggregates. We showed that the problem arises when the lysosome is unable to dock properly with the autophagosome. We used cerebral VSMCs with R133C mutation in *NOTCH3* gene. To follow up on this study, we would need to establish several different cerebral VSMC lines containing other CADASIL mutations. CADASIL patients with C445R mutation, as described previously, have severe symptoms of CADASIL with early ischemic attacks. It would be interesting to investigate the accumulation and aggregation of NOTCH3 in VSMCs containing C445R NOTCH3 mutation. Based on our findings, we believe that VSMCs containing C445R NOTCH3 mutation should exhibit more aggregation and accumulation and far more autophagosomes compared to our current cell model. Currently, the issue of NOTCH3 aggregation and accumulation and its pathological role in CADASIL has not been fully described. We believe that if we could show correlation between the different CADASIL mutations and an increase in autophagosomes and disease progression, we could potentially demonstrate that aggregation of NOTCH3 is the pathological driver in CADASIL.

Next, we investigated glucose uptake in CADASIL VSMCs and compared it control VMSCs. We noticed that glucose transporters are down regulated in CADASIL VSMCs relative to control VSMCs and that this down regulation had a functional effect in the sense that CADASIL VSMCs were not able to take up glucose as well as control VSMCs. One transporter that was down regulated was GLUT4, which is the only insulin-dependent transporter in our data set. Experimental set up was performed by adding insulin to the media and measuring the total glucose uptake in CADASIL VSMCs and control VSMCs. Insulin increased glucose uptake to some extent, which supports the notion that VSMCs utilize more than GLUT4 for their glucose uptake. It should be noted that the insulin-independent transporters were also down regulated but could not be rescued with insulin.

Staining for glucose transporters in post mortem brain tissues from CADASIL patients confirmed our cell model and allowed visualization of the down regulation of glucose transporters in CADASIL cerebral vessels compared to control tissues. An interesting observation was that larger cerebral vessels did not exhibit low levels of glucose transporters to the same extent as smaller vessels. Some CADASIL mutations produce symptom manifestation and signs of the disease earlier than others, suggesting that those mutations are more harmful. As a follow-up study, it would be interesting to use brain tissues with different CADASIL mutations and observe glucose transporters in those samples. One would suspect that the more harmful the mutation is, the less glucose transporters will be observed on VSMCs in post mortem tissues. Also, it would

be interesting to find subject with CADASIL and diabetes and measure the glucose uptake in the brain via PET and, if possible, obtain brain tissues to measure the abundance of glucose transporters in both the brain tissue and cerebral VSMCs. It is possible that comorbidity of diabetes with CADASIL would exacerbate the CADASIL disease progression.

In a subsequent study, we focused on the inflammatory role in CADASIL by measuring pro-inflammatory markers in CADASIL VSMCs. We hypothesized that, since CADASIL VSMCs are less resistant to stress and are generally weaker than their counterparts, they may produce and secret inflammatory cytokines as a "cry for help" to recruit other cells or molecules. The experiment was designed to measure gene expression of pro-inflammatory cytokines in CADASIL and control VSMCs. Pro-inflammatory gene expression in CADASIL VSMCs was overall higher compared to controls, with ICAM-1 and IL-6 having the highest expression. Co-culturing CADASIL VSMCs with ECs caused ECs to have a higher IL-12 and IL-1 expression, suggesting that CADASIL VSMCs cause an inflammatory response in their neighboring cells. This project is still in its infancy and we have planned on using frozen post mortem brain tissues from CADASIL to investigate cytokine levels in the brain. We will also investigate the number of activated microglial cells as a response to inflammatory elements in the brain. We hypothesize that we should see more activated microglia in close proximity to cerebral blood vessels in CADASIL tissue as VSMCs produce pro-inflammatory signals.

In these studies, we have tried to illustrate the multitude of events that can occur in CADASIL disease progression. Inhibition of any one of these events has the potential to slow progression of the disease or even halt progression. However, these studies are obviously preliminary and animal studies as well as VSMCs carrying other CADASIL mutations are needed to verify and further strengthen our reports.

6.1 LIMITATIONS OF THIS THESIS

Although CADASIL is generally a good model to study SVD, it is difficult to work with. Throughout this thesis, many challenges arose that limited our ability to fully investigate this disease in greater detail.

One of the more difficult tasks we encountered was that VSMCs are hard to genetically manipulate. We tried several transfection methods, including electroporation and plasmid transfection, but the VSMCs would either die out or lose the plasmid insert before stably expressing the mutated NOTCH3. We also noticed some subtle differences in the morphology of VSMCs obtained from different locations in the body. Aortic VSMCs were generally larger and exhibited a proliferation rate higher than that of umbilical or cerebral VSMCs.

In studies where we used post mortem tissues, great care was applied to find gender- and age-matched tissues. However, there are several drawbacks that come with using post mortem tissues. One problem is collection of brain tissue after death. Several factors can affect the tissue, such as body temperature before collection, time lapse between time of death and collection, and fixation of the tissue. Many biochemical events occur in the brain at death, which leads to degradation of tissues and release of chemicals that can alter experimental outcomes. Therefore, it is important that the brain is harvested as soon as possible after death. Another caveat in using human tissue in CADASIL is that many variables have an effect on the human body such as diet, exercise, drug use, and other lifestyle choices – making control of such variables impossible. Even with the challenges stated above, human tissue it is still one of the best models to study CADASIL.

In closing, as our understanding of CADASIL matures, additional questions arise regarding the exact mechanisms of pathology. Our work over the past few years has shed light on several aspects of CADASIL that have not been previously investigated, and has implicated several processes that may exacerbate disease progression. For these reasons, it is important that continued studies be undertaken to elucidate the mechanisms underlying CADASIL disease and its progression.

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